Cancer Chemother Pharmacol (2006) 57: 591–598
DOI 10.1007/s00280-005-0076-6

ORIGINAL ARTICLE

**Haifeng Song · Jin Zhang · Zhikai Han
Xueyan Zhang · Zheng Li · Lisheng Zhang
Ming Fu · Chen Lin · Jie Ma**

Pharmacokinetic and cytotoxic studies of pegylated liposomal daunorubicin

Received: 4 April 2005 / Accepted: 6 July 2005 / Published online: 30 August 2005

Abstract Pegylated liposomes have been studied for nearly two decades. However, fewer pharmacological studies about its application in daunorubicin (DNR) than those in doxorubicin have been reported. In order to conduct a complete pharmacokinetic study, radiolabeled DNR was encapsulated in pegylated liposomes. Its in vitro drug release kinetics was determined to be in a slow manner, which was reflected in its cytotoxic effect on four cell lines. The lethal dose, plasma pharmacokinetics as well as tissue distribution of the formulation were evaluated in comparison with free DNR. The results revealed that liposomal daunorubicin significantly reduced the toxicity of the drug, with a half lethal dose of 29.35 mg/kg, compared with 5.45 mg/kg for free drug. Pharmacokinetic study of liposomal DNR demonstrated a slower clearance rate, an elevated area under the concentration–time curve, as well as increased half-lives compared to free drug. In addition, an altered tissue distribution of liposomal DNR was observed, with lower cardiac accumulation. Taken together, pegylated liposome-loaded DNR may be a promising anticancer drug and worth further therapeutic study.

Keywords Daunorubicin · Liposome · Pharmacokinetic

H. Song · Z. Han · X. Zhang · Z. Li · M. Fu · C. Lin · J. Ma (✉)
State Key Laboratory of Molecular Oncology,
Cancer Institute, Chinese Academy of Medical Sciences,
PUMC, Beijing 100021, China
E-mail: clin@public.bta.net.cn
Tel.: +86-10-67723793
Fax: +86-10-87714054
E-mail: jiema2000@yahoo.com
Tel.: +86-10-87788885
Fax: +86-10-87788451

J. Zhang · L. Zhang
Center for Drug Preclinical Evaluation, Cancer Institute,
Chinese Academy of Medical Sciences,
PUMC, Beijing 100021, China

Introduction

Daunorubicin (DNR) is an anthracycline antibiotic originally obtained from *Strptomyces peucetius* with documented anti-tumor activity in a variety of malignancies, such as leukemia, lymphoma, neuroblastoma, nephroblastoma, Ewing's sarcoma and other solid tumors [25]. DNR has activity comparable to doxorubicin in patients with leukemia and lymphoma but may be less effective when treating solid tumors. Despite its extensive use clinically, DNR has been largely circumvented by its dose-limiting toxicities, such as myelosuppression, mucositis, gastrointestinal toxicities, alopecia and more importantly, cardiotoxicity.

In order to reduce the toxic effects of DNR and improve its therapeutic efficacy, drug carriers are often desired. The ideal carrier should be nontoxic, biodegradable and specific for the target site where active contents are released. Liposomes have long been considered as the ideal carriers for anti-cancer drugs in attempt to deliver active agents to tumors in vivo, to relieve sensitive tissues from toxicity, or to protect their entrapped contents from degradation, thus resulting in improved therapeutic index and/or reduced toxicity [2]. Moreover, sustained drug release property is one of the benefits of the use of liposomes as drug carriers.

Sterically stabilized liposomes (SSLs) characterized by polymeric polyethylene glycol (PEG) coating altered pharmacokinetics and biodistribution of various drugs in a large number of studies [1, 6, 7, 20– 24, 27]. In comparison to conventional liposomes without PEG modification, these pegylated SSLs showed a prolonged retention time in blood, primarily due to the reduced recognition and uptake by phagocytic cells of the mononuclear phagocytic system (MPS) located mainly in the liver and spleen. Thus, they are less readily cleared from circulation. Furthermore, PEG coating has been believed to prevent liposome clearance by neutralizing the surface charge of liposomes and shielding various opsonins in the plasma from binding. These properties

would reduce the interactions between liposomes and plasma proteins or cell surface receptors and enable pegylated liposomes to be more promising drug carriers compared to conventional liposomes [1, 2, 21]. Indeed, there is one pegylated liposome drug formulation, SSL-doxorubicin (Doxil), currently available [6, 7]. A number of investigations in either animal models or patients, with DNR incorporated into conventional liposomes, have demonstrated reduced toxicity and maintained or enhanced anti-tumor effects [9–13, 15, 16, 28]. However, there are very few reports on the DNR encapsulated into SSLs.

Therefore, our objectives in this study are to evaluate the stability and release kinetics in vitro of the encapsulated DNR in pegylated SSLs as well as pharmacokinetics and tissue distribution of this formulation so as to establish a more ideal DNR carrier system. In order to enhance the encapsulation efficiency of DNR into liposomes, we employed remote loading method, in which the entering of drug molecules into the liposome interior was driven by an ammonia sulfate gradient [5]. We investigated the sterically stabilized pegylated liposomes containing DNR in terms of in vitro plasma release rate, in vivo lethal dose (LD_{50}), pharmacokinetics and biodistribution with respect to free DNR. Reduced toxicity, improved pharmacokinetics and altered biodistribution have been elucidated.

Materials and methods

Chemicals

Daunorubicin was purchased from Pharmacia, China. Tritium-labeled DNR, ^{3}H-DNR, was customer designed from China Institute of Nuclear Energy with the specific activity of 200 μCi/mg (0.74 mBq/mg). Hydrogenated soybean phosphatidylcholine (HSPC) and polyethylene glycol-distearoylphosphatidylethanolamine (PEG_{2000}-DSPE) were purchased from Lipoid (Lipoid GmbH Ludwigshafen, Germany). Cholesterol was obtained from Sigma Chemical Co. (St Louis, MO, USA). All chemicals used in this study were in analytical grade purity.

Cell lines

Human lung adenocarcinoma cell line A549, colon cancer cell line HCT8 and acute myeloid leukemic cell line HL60 were purchased from ATCC. Human hepatocarcinoma cell line BEL7402 was derived from Shanghai Institute of Cell Bilolgy (Cell Institute of Chinese Academy of Science). All cell lines were cultured in RPMI-1640 medium (GIBCO) supplied with 10% heat-inactivated fetal calf serum (FCS) for cytotoxic study.

Mice

Kunming female mice of 25 ± 2 g in weight were provided by Animal Institute, Chinese Academy of Medical Sciences. All mice were housed with free access to water and food.

Preparation of liposomes

Liposome formulations were prepared by thin lipid film hydration as previously described with minor modifications. In brief, lipid mixtures composed of HSPC, cholesterol and PEG–DSPE in a molar ratio of 52:40:8 were dissolved in chloroform solution, evaporated to dryness under reduced pressure in a round-bottom glass flask. The lipid film was then hydrated with 1 ml of 250 mM ammonium sulfate, pH 5.5, followed by sequential extrusion using an extruder (Avestin Inc., Ottawa, Canada) repeatedly through 200, 100 and 80 nm pore size polycarbonate membranes (Nuclepore, Pleasanton, CA, USA), to produce primarily unilamellar vesicles at 65°C and under nitrogen pressure of 200–500 psi. The external buffer was then exchanged by passing the liposome suspension though a Sephadex G-50 column equilibrated with 10% (w/v) sucrose solution.

Drug loading

Daunorubicin or ^{3}H-DNR was loaded into liposomes via an ammonium sulfate gradient as reported previously [5]. Daunorubicin or ^{3}H-DNR was dissolved in 10% (w/v) sucrose, and then the drug solution was incubated with liposomes at 65°C for 15 min with the drug/lipid ratio of 0.2:1 (wt/wt). The unencapsulated drug was removed from the liposome suspension by passing through a Sephadex G-50 column eluted with phosphate-buffered saline (PBS), pH 7.4. This DNR–liposome formulation was sterilized by filtration through 0.22 μm pore cellulose membranes (Millipore, Bedford, MA, USA) and stored at 4°C for future use. The average diameter of the liposomes was measured by dynamic laser light scattering using a Brookhaven BI90 submicron particle size analyzer (Brookhaven Instrument Corp., Holtsville, NY, USA). Lipid and drug concentration were examined spectrophotometically. DNR concentration in the liposomes was calculated from absorbance at 478 nm following dissolution in 4% Triton X-100. Phospholipid concentration was determined as previously described by Bartlett [4]. Liposome solutions were stored at 4°C for further study.

In vitro drug release study

Kinetics of drug efflux from the liposomes was studied by incubating the ^{3}H-DNR-containing liposomes at 37°C with either 25% human plasma or PBS (pH 7.4) at

a ratio of 1:9 (v/v). Briefly, before the release experiment, SSL–DNR was passed through a G50 column to separate free DNR from SSL–DNR. Then the above solutions of liposomes (5 ml) were deposited in dialysis sacs (M. Wt. cutoff 6,000 kDa) and dialyzed against either 25% human plasma or PBS at 37°C, respectively. Aliquots (100 μl) from each depot were withdrawn at designated time points (0.5, 2, 4,6, 8, 12, 24, 36, 48 and 72 h) to measure the radioactivity by a Beckman β-liquid scintillation counter (LS 5801, Beckman). The radioactivity of drug efflux from the liposomes was quantitatively evaluated as the drug release rate.

In vitro cytotoxicity

Cytotoxicity of SSL–DNR was determined by 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide assay (MTT). Adhere cells (A549, HCT8 and BEL7402) were plated at 5×10^4 cells/well and suspension cells (HL60) plated at 2×10^5 cells/well in 96-well plates, respectively. After a 24 h culture, free DNR or SSL–DNR were added into the wells at final DNR concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 μg /ml. The cell growth was evaluated in 24, 48 and 72 h by MTT assay. Briefly, MTT dye was added to each well at a final concentration of 0.5 mg /ml. After a 4-h incubation, the media was replaced by 100 μl dimethylsulfoxide (DMSO) to solubilize formazan complex. Optical density at 490 nm was determined using a EL312e microplate bio-kinetics reader (Bio-tek instruments, Inc., USA).

Pharmacokinetics and tissue distribution studies

Forty-two Kunming mice were randomized into seven groups. Radiolabeled DNR, either in free form or in liposome-associated form, was injected via the tail vein to the mice with a single dose of 2 mg/kg body weight (10 mg/kg of phospholipid) in 0.2 ml using 29-gauge needles. At the indicated time points after the injection (0.5, 2, 6, 12, 24, 48 and 72 h), three mice were anesthetized by ether inhalation and sacrificed by cervical dislocation at each time point. Twenty microliters of blood were collected by heart puncture from each mouse. Organs such as the liver, heart, kidney, lungs and spleen were immediately removed, rinsed with saline and dried. The blood samples and 10 mg of tissues from each organ were incubated with 20 μl of 60% $HClO_4$ and 40 μl of H_2O_2 at 70–80°C for 30–60 min until the samples appeared clear. Then the digestion was terminated and cooled down to room temperature. One milliliter of 0.6% ppo methyl bezene and 0.6 ml glycol methyl-ether were added into the samples prior to the liquid scintillation counting. Radioactivity of tissue samples was measured with a Beckman β-liquid scintillation counter (LS 5801, Beckman).

Toxicity

The lethal dose of DNR and liposomal DNR was determined by administrating free DNR and SSL–DNR with increasing doses into Kunming mice, respectively. Ten mice were used in each dose group. The doses of intraperitoneal injections were elevated as 2.4, 3.4, 4.9, 7 and 10 mg/kg body weight for free DNR (Dm = 10 mg/kg, k = 0.7) as well as 12.6, 18, 25.7, 36.7 and 52.5 mg/kg body weight for SSL–DNR (Dm = 52.5 mg/kg, k = 0.7), respectively. Mice were closely monitored for 14 days.

Statistics

Results are presented as mean ± SD. LD_{50}, pharmacokinetics and tissue distribution were calculated by using Microsoft Excel 2000 and DAS 1.0 (Drug and Statistics for Windows, Anhui Provincial Center for Drug Clinical Evaluation, China).

Results

Characterization of liposomes

The resulting liposomes had a mean diameter of 110 ± 10 nm. The loading efficiency was more than 95%. DNR entrapped in the liposomes at a concentration of 150–180 μg DNR/ μmol phospholipids.

Release study of SSL-DNR

Figure 1 shows that SSL-DNR exhibited similar stability when incubated either in 25% human plasma or PBS

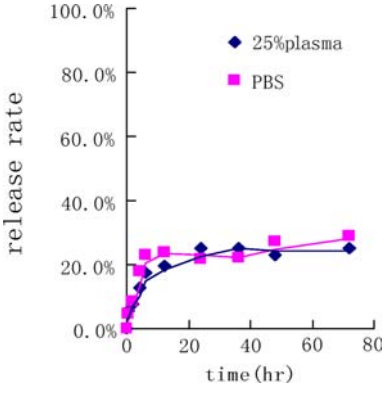

Fig. 1 Drug release profile of SSL-DNR. Kinetics of drug efflux from the liposomes was studied by incubating the ^{3}H-DNR-containing liposomes at 37°C with either 25% human plasma or phosphate-buffered saline (PBS, pH 7.4) at a ratio of 1:9 (v/v). Then the above solutions of liposomes (5 ml) were deposited in dialysis sacs (M.Wt. cutoff 6,000 kDa) and dialyzed against either 25% human plasma or PBS at 37°C respectively. Aliquots (100 μl) from each depot were withdrawn at designated time points (0.5, 2, 4,6, 8, 12, 24, 36, 48 and 72 h) to measure the radioactivity by a Beckman β-liquid scintillation counter (LS 5801, Beckman)

at 37°C. The dissociated DNR from the liposomes during 72-h-experimental period in human plasma and PBS were 25.1 and 29.1% of total DNR encapsulated, respectively. The release of DNR has a burst release phase at the beginning, approximately 20% of the drug released at first 12 h and the release rate reduced thereafter, which indicated that the release of DNR reached a slow release status. Thus a depot effect could be achieved using SSL, which released its contained drug at a slow speed. According to the experimental results of SSL-cisplatin from Zamboni et al.[27] and Newman et al.[20], there was almost no platinum released from SSL in vitro. This raised a problem that the drug was too stably associated with liposomal carrier to be released in vivo, which might influence the effectiveness of the drug. In our experiment, we observed slow release of DNR in vitro. This meets the requirement of the delivery system, which keeps the drug stable in the circulation and releases the drug slowly at tumor site.

Cytotoxic assay

The cell lines used for cytotoxic assay were in the logarithmic phase. The dose–response curves are shown in Fig 2 and the data calculated from the curves are summarized in Table 1. The data showed that for cell lines BEL7402 and HCT8, the IC_{50} of free DNR was much lower than SSL-DNR. After a 24 h reaction, there no detectable toxicity of SSL-DNR. This is consistent with our release study, because the killing activity depends on the quantity of drug released. Thus the longer the

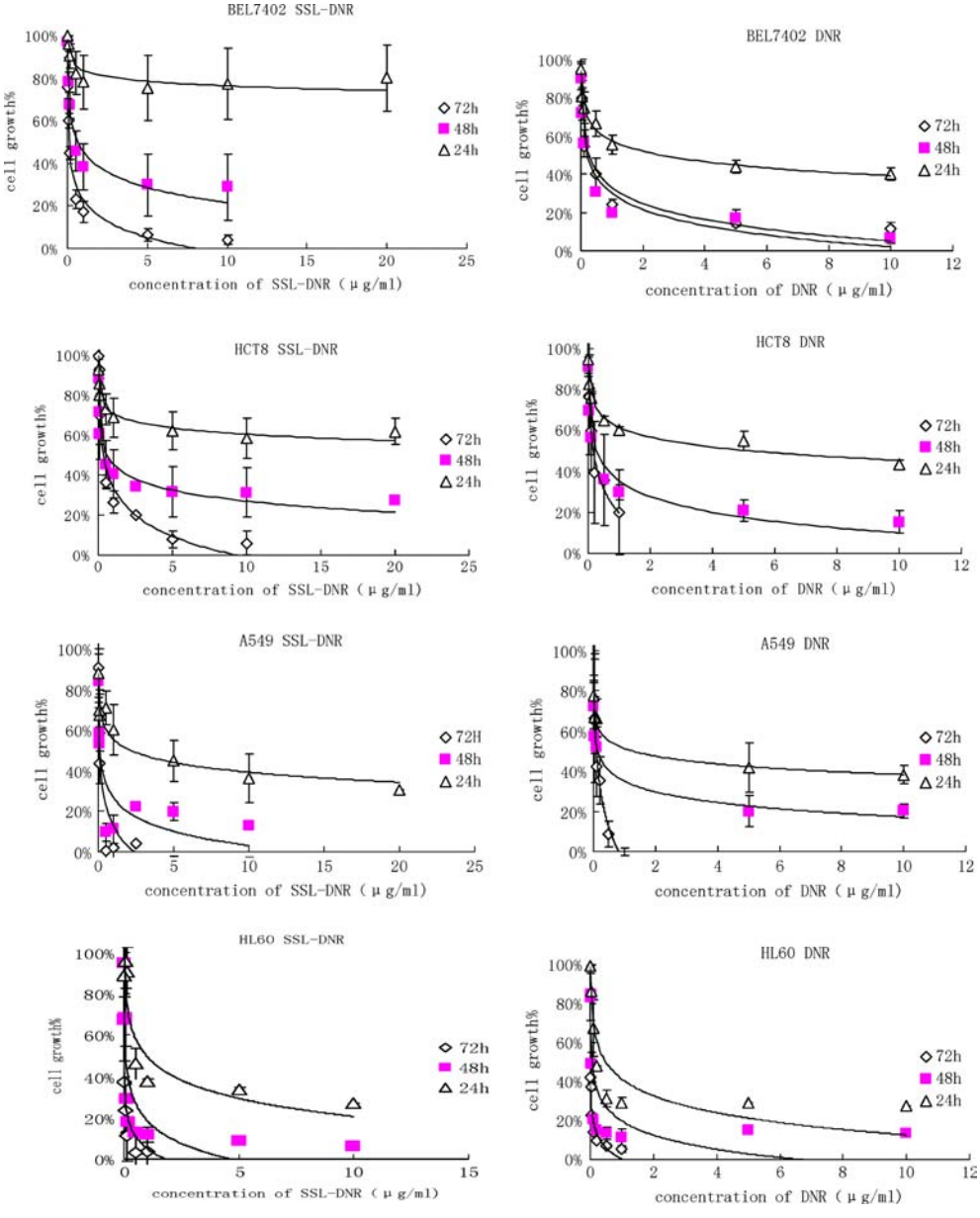

Fig. 2 Cytotoxicity of SSL-DNR and DNR on four tumor cell lines determined by MTT assay. Adhere cells (A549, HCT8 and BEL7402) were plated at 5×10^4 cells/well and suspension cells (HL60) plated at 2×10^5 cells/well in 96-well plates respectively. After a 24 h culture, free DNR or SSL-DNR was added into the wells at final DNR concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 μg /ml. The cell growth was evaluated in 24, 48 and 72 h by MTT assay. The dose–response curves showed the cytotoxicity of SSL-DNR based on the release of DNR from liposomes

Table 1 Inhibition dose 50 (IC_{50}, μg /ml) derived from cytotoxicity assay (MTT test) for free DNR and liposomal DNR (SSL-DNR)

	Cell lines IC_{50}(μg/ml)							
	BEL7402		HCT8		A549		HL60	
	DNR	SSL-DNR	DNR	SSL-DNR	DNR	SSL-DNR	DNR	SSL-DNR
24 h	2.595	∞	5.222	∞	0.498	2.129	0.498	0.981
48 h	0.196	0.631	0.259	0.565	0.145	0.101	0.056	0.077
72 h	0.067	0.105	0.169	0.349	0.004	0.018	0.003	0.005

The IC_{50} was calculated from the dose–response curves of the cell lines, bel7402, HCT8, A549 and HL60

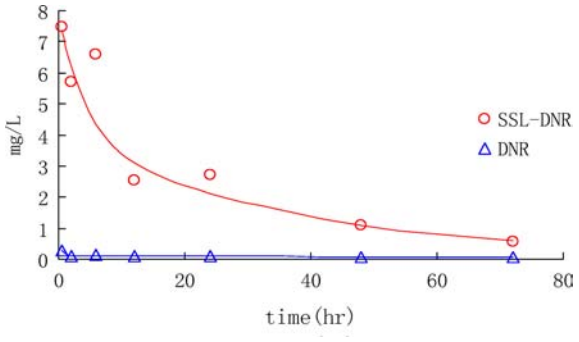

Fig. 3 Plasma pharmacokinetics of free DNR and SSL-DNR in Kunming mice. Values were means for three determinations. SSL-DNR showed a prolonged retention in the plasma in a bi-exponential manner

reaction time, the minor the difference of IC_{50} between DNR and SSL-DNR. The cell lines A549 and HL60 were quite sensitive to SSL-DNR, which was reflected by their low IC_{50}. These two cell lines could be considered for further animal study.

Plasma pharmacokinetics

SSL-loaded DNR and free DNR were administered intravenously to Kunming mice via the tail vein at equivalent doses of 2 mg DNR/kg body weight in an injection volume of 200 μl. The clearance kinetics of the DNR from plasma was determined by measuring the cpm of radiolabeled DNR in either free or encapsulated form. As shown in Fig. 3, free DNR was rapidly cleared from the plasma in a biphasic behavior. Corresponding plasma half-lives were 0.1 ($t_{1/2\alpha}$)and 12 h ($t_{1/2\beta}$), respectively (see Table 2). On the contrary, SSL-DNR showed a prolonged retention in the plasma in a bi-exponential manner with the half-life being 2.9 ($t_1/2\alpha$)and 26 h ($t_1/2\beta$). These half-life data were consistent with an early observation of SSL–doxorubicin [14]. The area under the time–concentration curve (AUC) for SSL-DNR was 27-fold increased compared with free DNR. Seventy-two hours after administration, a large amount of DNR remained detectable in the plasma for the SSL-DNR with the concentration of 1.2 mg/l. Moreover, a slower clearance and a smaller volume of distribution were found for SSL-DNR compared with free DNR (Table 2).

Tissue distribution of DNR

After a single i.v. injection of 2 mg/kg body weight SSL-DNR as well as an equivalent dose of free DNR, the tissue distribution of DNR was determined by measuring DNR radioactivity in various organs. As shown in Fig. 4, the liver and spleen were the organs with the highest accumulation of SSL-DNR. On the other hand, the accumulative concentration of SSL-DNR in heart was significantly lower than that of free drug, which was consistent with the characteristics of SSL-doxorubicin or SSL-cisplatin [3, 20]. The drug concentration in lungs and kidney for both formulations was comparable. The limitation of this method was that we could not distinguish the released parent drug and metabolites, some of which may be inactive, especially 24 h after injection.

Toxicity

A dose range finding study of the toxicity of DNR and SSL-DNR was conducted (Fig.5). The LD_{50} values for free DNR and SSL-DNR were 5.45 and 29.35 mg/kg body weight, respectively. Thus, SSL-DNR considerably reduced the toxicity of the drug.

Discussion

The ability of liposomes to preferentially accumulate at disease sites, in particular tumor as well as infection and inflammation, has led to their development as drug

Table 2 Pharmacokinetic parameters for DNR and SSL-DNR after intravenously injected in Kunming mice at dose of 2 mg/kg

	$T_{1/2}$(h)	$AUC_{0-72\ h}$(mg/l×h)	C_{max}(mg/l)	Clearance(ml/h)	V_d (ml)
Free DNR	α = 0.113 β = 12.105	5.839	0.268	0.0425	4.906
SSL-DNR	α = 2.919 β = 25.986	159.204	7.463	0.003	0.129

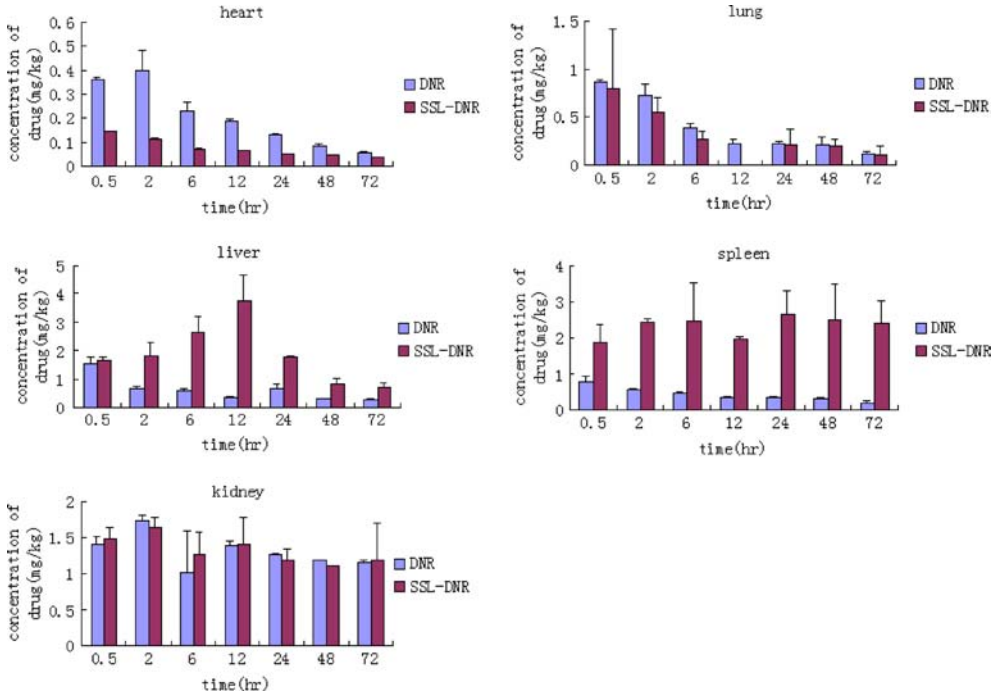

Fig. 4 Tissue distribution of DNR or SSL-DNR after i.v. injection. The tissue distribution of DNR was determined by measuring DNR radioactivity in various organs

carriers. This selective accumulation is believed to reflect extravasation of liposomes from the discontinuous or damaged vasculature, which significantly increases the local permeability of the drug [26]. The extent of accumulation within the tumor is largely determined by the circulation lifetime of the carriers. In this regard, carrier systems which display higher levels of drug retention will provide greater tumor delivery. Coating with PEG to the liposome bilayer provides a highly hydrophilic shield for the liposomes from electrostatic and hydrophobic interactions with serum components as well as reduced uptake by MPS. These permit PEG-coated liposomes to display prolonged blood residence time, improved pharmacokinetic profile and altered biodistribution. In this study, in comparison with free DNR, SSL-DNR showed increased half-lives, augmented AUC, lower volume of distribution and decreased clearance rate.

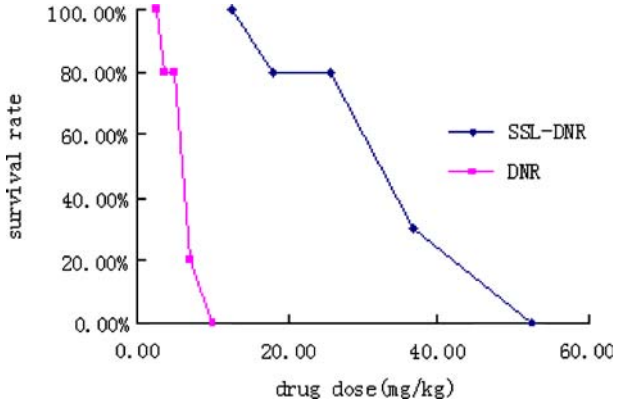

Fig. 5 The toxicity of DNR or SSL-DNR in mice. The doses of intraperitoneal injections were elevated as 2.4, 3.4, 4.9, 7 and 10 mg /kg body weight for free DNR as well as 12.6, 18, 25.7, 36.7 and 52.5 mg /kg body weight for SSL-DNR, respectively. Mice were closely monitored for 14 days

The parameters of pharmacokinetics and tissue distribution of SSL-DNR were similar to those of its analogue, SSL-doxorubicin, which indicated that encapsulated drug contents have little impact on the liposome [14]. This was in good agreement with the speculation in that the plasma pharmacokinetics and tissue distribution of the encapsulated materials were characterized by the liposome itself, but not the internalized drug, i.e. the behavior of the SSL–liposomes was hardly changed regardless of their encapsulated contents.

The volume of distribution of SSL-DNR practically approached that of the mouse plasma volume. This indicated that liposomal encapsulated drug molecules were confined primarily to the blood compartment rather than various tissues [2]. Lasic et al. [19] pointed out that encapsulated anthracyclines within SSLs are in stabilized conformation, in that the drug molecules precipitate in the interior of liposomes as a gel-like structure. The DNR loaded within SSLs may also be in such a stabilized structure. Our in vitro drug efflux study showed that the encapsulated drug molecules were slowly released out of the SSL with the leaking rate of DNR determined at 72 h lying in the range of 20–30%. Our previous study showed that the release rate of doxorubicin (DXR) from SSLs was in a comparably slow manner with release rate less than 10% (data not shown). This difference may be due to a more hydrophobic character of DNR than DXR, which led to a slightly faster leakage. The different evaluating methods might also contribute to this difference. In this study, the

leaking rate was determined by the radioactivity of DNR, which was much more sensitive than optical density reading used for DXR leakage study. Since the addition of PEG forms a protecting polymeric layer by flexible polymeric chain on the surface of liposomes, which prevents protein adsorption, we did not observe a faster leakage of DNR from liposomes suspended in serum compared to protein-free buffer. In an early study conducted by Huwyler et al.[18] tritium-labeled DNR was entrapped within SSLs and pharmacokinetics and tissue distribution were assessed in Sprague-Dawley rats. They found free DNR was rapidly cleared from the circulation which was in good agreement with our observation. However, some of the biodistribution data of SSL-DNR were conflicting with our observation. For example, the AUC of SSL-DNR was 285-fold increased compared to that of free DNR, which was much higher than our data. In the tissue distribution study, these authors indicated that the drug accumulation was lowered not only in heart, which was consistent with our findings, but also in other tissues, e.g. liver, spleen, kidney, lungs. The discrepancies resulted from a short observation period of the early study, since tissue distribution of 60 min is irrelevant for a formulation with a half-life of 12 h. Higher accumulation of SSL-DNR in spleen and liver might result from the tendency of SSL uptake by MPS organs and relatively higher vascular permeability in these organs.

The reduced toxicity of DNR is benefited from altered liposome distribution, especially in heart. The in vivo acute toxicity study indicated that SSL-DNR dramatically reduced the drug toxicity with LD_{50} of 29.35 mg/kg, which was five folds more than that of free DNR. This result was obtained from a toxicity study using i.p. injection. Since liposomes enter the circulatory system via lymphatic drainage from the peritoneum, a process that is much slower than for free DNR, this injection route might magnify the difference between the free drug and its liposomal formulation. However, we believe that this limitation could not erase the significance between these two LD_{50}values. This reduced toxicity of SSL-DNR is attributed to its slow release properties as well as liposome redistribution.

Our in vitro cytotoxicity assays with either free DNR or SSL-DNR against several different cell lines demonstrated that the liposome-encapsulated drug was much less potent than that of free drug, as IC_{50} increased multiple fold for SSL-DNR. This has been ascribed to the fact that the majority of drug molecules remained entrapped at the aqueous core of liposome and restrained by liposomes for interaction with cells, thus only a small fraction of the drug molecules have been released out of the liposomes and subsequently taken up by the cells. This decrease of the in vitro cytotoxicity effect with respect to encapsulation is in good agreement with previous observations [8, 17].

Taken together, pegylated liposmal DNR showed improved pharmacokinetic profile, altered tissue distribution and reduced toxicity. This formulation may provide a good candidate in the chemotherapeutic treatment against a variety of tumors. The therapeutic effects of SSL-DNR in vivo in several different tumor models are under investigation in our laboratory.

Acknowledgements This project was supported by the Chinese Hi-Tech R &D program of China (No: 2002AA412131). Principal investigator: Jie Ma, MD PhD.

References

1. Allen TM (1994) Long-circulating (sterically stabilized) liposomes for targeted drug delivery. Trends Pharmacol Sci 15:215–220
2. Allen TM, Cullis PR (2004) Drug delivery systems: entering the mainstream. Science 303:1818–1822
3. Bandak S, Goren D, Horowitz A, Tzemach D, Gabizon A (1999) Pharmacological studies of cisplatin encapsulated in long-circulating liposomes in mouse tumor models. Anticancer Drugs 10:911–920
4. Bartlett GR (1959) Phosphorus assay in column chromatography. J Biol Chem 234:466–468
5. Bolotin EM CR, Bar LK, Emanuel SN, Lasic DD, Barenholz Y (1994) Ammonium sulphate gradient for efficient and stable remote loading of amphopathic weak bases into liposomes and ligandoliposomes. J Liposome Res 4:455–479
6. Colbern GT, Hiller A, Musterer RS, Pegg E, Henderson IC, Working P (1999) Significant increase in antitumor potency of doxorubicin HCl by its encapsulation in pegylated liposomes. J Liposome Res 9:523–538
7. Colbern GT, Vaage J, Donovan D, Uster P, Working P (2000) Tumor uptake and therapeutic effects of drugs encapsulated in long-circulation pegylated stealth® liposomes. J Liposome Res 10:81–92
8. Drummond DC, Meyer O, Hong K, Kirpotin DB, Papahadjopoulos D (1999) Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. Pharmacol Rev 51:691–743
9. Eckardt JR, Campbell E, Burris HA, Weiss GR, Rodriguez GI, Fields SM, Thurman AM, Peacock NW, Cobb P, Rothenberg ML et al (1994) A phase II trial of DaunoXome, liposome-encapsulated daunorubicin, in patients with metastatic adenocarcinoma of the colon. Am J Clin Oncol 17:498–501
10. Eucker J, Eikel D, Heider U, Jakob C, Zavrski I, Gatz F, Mergenthaler HG, Jungclas H, Possinger K, Sezer O (2003) Liposomal daunorubicin (DaunoXome) in multiple myeloma: a modified VAD regimen using short-term infusion. Anticancer Drugs 14:793–799
11. Fassas A, Buffels R, Anagnostopoulos A, Gacos E, Vadikolia C, Haloudis P, Kaloyannidis P (2002) Safety and early efficacy assessment of liposomal daunorubicin (DaunoXome) in adults with refractory or relapsed acute myeloblastic leukaemia: a phase I–II study. Br J Haematol 116:308–315
12. Forssen EA, Coulter DM, Proffitt RT (1992) Selective in vivo localization of daunorubicin small unilamellar vesicles in solid tumors. Cancer Res 52:3255–3261
13. Forssen EA, Male-Brune R, Adler-Moore JP, Lee MJ, Schmidt PG, Krasieva TB, Shimizu S, Tromberg BJ (1996) Fluorescence imaging studies for the disposition of daunorubicin liposomes (DaunoXome) within tumor tissue. Cancer Res 56:2066–2075
14. Gabizon A, Catane R, Uziely B, Kaufman B, Safra T, Cohen R, Martin F, Huang A, Barenholz Y (1994) Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. Cancer Res 54:987–992
15. Guaglianone P, Chan K, DelaFlor-Weiss E, Hanisch R, Jeffers S, Sharma D, Muggia F (1994) Phase I and pharmacologic study of liposomal daunorubicin (DaunoXome). Invest New Drugs 12:103–110

16. Hempel G, Reinhardt D, Creutzig U, Boos J (2003) Population pharmacokinetics of liposomal daunorubicin in children. Br J Clin Pharmacol 56:370–377
17. Horowitz AT, Barenholz Y, Gabizon AA (1992) In vitro cytotoxicity of liposome-encapsulated doxorubicin: dependence on liposome composition and drug release. Biochim Biophys Acta 1109:203–209
18. Huwyler J, Yang J, Pardridge WM (1997) Receptor mediated delivery of daunomycin using immunoliposomes: pharmacokinetics and tissue distribution in the rat. J Pharmacol Exp Ther 282:1541–1546
19. Lasic DD, Ceh B, Stuart MC, Guo L, Frederik PM, Barenholz Y (1995) Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery. Biochim Biophys Acta 1239:145–156
20. Newman MS, Colbern GT, Working PK, Engbers C, Amantea MA (1999) Comparative pharmacokinetics, tissue distribution, and therapeutic effectiveness of cisplatin encapsulated in long-circulating, pegylated liposomes (SPI-077) in tumor-bearing mice. Cancer Chemother Pharmacol 43:1–7
21. Papahadjopoulos D, Allen TM, Gabizon A, Mayhew E, Matthay K, Huang SK, Lee KD, Woodle MC, Lasic DD, Redemann C et al (1991) Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci USA 88:11460–11464
22. Parr MJ, Ansell SM, Choi LS, Cullis PR (1994) Factors influencing the retention and chemical stability of poly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles. Biochim Biophys Acta 1195:21–30
23. Peleg-Shulman T, Gibson D, Cohen R, Abra R, Barenholz Y (2001) Characterization of sterically stabilized cisplatin liposomes by nuclear magnetic resonance. Biochim Biophys Acta 1510:278–291
24. Williams SS, Alosco TR, Mayhew E, Lasic DD, Martin FJ, Bankert RB (1993) Arrest of human lung tumor xenograft growth in severe combined immunodeficient mice using doxorubicin encapsulated in sterically stabilized liposomes. Cancer Res 53:3964–3967
25. Young RC, Ozols RF, Myers CE (1981) The anthracycline antineoplastic drugs. N Engl J Med 305:139–153
26. Yuan F, Leunig M, Huang SK, Berk DA, Papahadjopoulos D, Jain RK (1994) Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. Cancer Res 54:3352–3356
27. Zamboni WC, Gervais AC, Egorin MJ, Schellens JH, Zuhowski EG, Pluim D, Joseph E, Hamburger DR, Working PK, Colbern G, Tonda ME, Potter DM, Eiseman JL (2004) Systemic and tumor disposition of platinum after administration of cisplatin or STEALTH liposomal-cisplatin formulations (SPI-077 and SPI-077 B103) in a preclinical tumor model of melanoma. Cancer Chemother Pharmacol 53:329–336
28. Zucchetti M, Boiardi A, Silvani A, Parisi I, Piccolrovazzi S, D'Incalci M (1999) Distribution of daunorubicin and daunorubicinol in human glioma tumors after administration of liposomal daunorubicin. Cancer Chemother Pharmacol 44:173–176